

Valency conversion in the type 1 fimbrial adhesin of *Escherichia coli*

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Summary

FimH protein is a lectin-like adhesive subunit of type 1, or mannose-sensitive, fimbriae that are found on the surface of most *Escherichia coli* strains. All naturally occurring FimH variants demonstrate a conserved mannotriose-specific (i.e. multivalent) binding. Here, we demonstrate that replacement of residues 185–279 within the FimH pilin domain with a corresponding segment of the type 1C fimbrial adhesin FocH leads to a loss of the multivalent mannotriose-specific binding property accompanied by the acquisition of a distinct monomannose-specific (i.e. monovalent) binding capability. Bacteria expressing the monovalent hybrid adhesins were capable of binding strongly to uroepithelial tissue culture cells and guinea pig erythrocytes. They could not, however, agglutinate yeast or bind human buccal cells – functions readily accomplished by the *E. coli*-expressing mannotriose-specific FimH variants. Based on the relative potency of inhibiting compounds of different structures, the receptor binding site within monovalent FimH–FocH adhesin has an extended structure with an overall configuration similar to that within the multivalent FimH of natural origin. The monomannose-only specific phenotype could also be invoked by a single point mutation, E89K, located within the lectin domain of FimH, but distant from the receptor binding site. The structural alterations influence the receptor-binding valency of

the FimH adhesin via distal effects on the combining pocket, obviously by affecting the FimH quaternary structure.

Introduction

Lectins are a structurally diverse class of proteins found in most living organisms, ranging from animals and plants to bacteria and viruses, that bind carbohydrate molecules in a non-covalent and non-enzymatic fashion (Sharon and Lis, 1989). Although some lectins are capable of binding simple monosaccharides, the lectin-mediated biological recognition interactions typically occur via specific binding to complex oligosaccharide ligands (Rini, 1995). Oligosaccharide-specific recognition is provided by a multivalent mechanism of the lectin–receptor interaction as a result of the presence of either multiple subsites or subunits in the lectin's structure (Rini, 1995). It has been assumed that the multivalent receptor binding ensures high affinity and specificity for cell–cell or ligand–receptor interactions.

The FimH lectin is the 30 kDa adhesin of *Escherichia coli* type 1 fimbriae and represents the most common kind of bacterial adhesins known that are sensitive to inhibition by soluble D-mannose and derivatives (Klemm and Krogfelt, 1994). By virtue of the FimH lectin, *E. coli* strains are able to bind to mannosylated receptors on a great variety of host cells of epithelial and other origin (Beachey, 1981). However, commensal and pathogenic isolates express functionally distinct types of the FimH adhesin, which differ in receptor specificity and, consequently, in host cell tropism (Sokurenko *et al.*, 1995; 1997; 1998; Pouttu *et al.*, 1999). FimH variants of most commensal isolates exhibit typical multivalent lectin properties – they have rather weak affinities for monomannose (1M) residues, but have a strong capability to bind complex mannose structures, in particular those N-linked high-mannose type oligosaccharides that contain terminally exposed α 1–3, α 1–6-D-mannotriose (3M) units (Sokurenko *et al.*, 1995; 1997). The 3M-only specificity allows *E. coli* to bind buccal epithelial cells in the presence of a relatively high concentration of soluble mannose-containing components of saliva (Sokurenko *et al.*, 1998). This could be important for the transient colonization of the oropharyngeal mucosa that is thought to be a critical step for interhost *E. coli* transmission (Bloch *et al.*, 1992). On the other hand, most

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uropathogenic isolates express FimH variants that exhibit both multivalent 3M specificity and monovalent 1M specificity (Sokurenko *et al.*, 1995). This allows them to bind various types of N-linked oligosaccharides containing any number of terminally exposed mannosyl residues (Sokurenko *et al.*, 1997). Compared with the 3M-only specific FimH, variants with the dual 1M/3M specificity are able to bind to a much broader spectrum of eukaryotic cells, which in turn leads to increased pathogenic potential (Sokurenko *et al.*, 1995; 1997; 1998). Bacteria expressing dual 1M/3M-specific type 1 fimbriae adhere strongly to uroepithelial cells and, in mouse models of urinary tract infection (UTI), are capable of colonizing the urinary bladder at a much higher level than bacteria expressing 3M-only specific fimbriae (Sokurenko *et al.*, 1998). FimH variants of certain meningitis-causing strains are also capable of collagen binding (Pouttu *et al.*, 1999).

The primary structures of the naturally occurring FimH variants are highly conserved (about 99% identity), and the 1M/3M-specific FimH variants differ from the 3M-only specific variants in one or a few amino acids (Sokurenko *et al.*, 1994; 1995; 1998). The three-dimensional structure of FimH has been elucidated recently (Choudhury *et al.*, 1999). According to this work, the FimH protein is folded into two domains, an N-terminal carbohydrate-binding domain (residues 1–156) linked by a short tripeptide loop to a C-terminal fimbriae integration domain (residues 160–279). In a recent structure–function analysis of FimH, we found that alterations that enhance 1M binding are located in different regions of the FimH molecule and, surprisingly, distant from the identified carbohydrate-binding pocket (Schembri *et al.*, 2000). It is unclear how amino acid alterations, which dramatically enhance monovalent 1M binding of the FimH lectin, do not significantly affect its multivalent 3M-binding property. One might suggest that the substitutions enhancing 1M binding (i) affects the conformation of a receptor-interacting subsite within an extended combining site of FimH; (ii) modifies one of multiple, i.e. structurally separate, receptor binding sites; or (iii) affects the tertiary or quaternary structure of the FimH protein without directly altering the binding site(s) itself.

In addition to FimH, other bacterial lectin-like adhesins are known to use a multivalent receptor-binding mechanism, e.g. the P-fimbrial and K99-fimbrial adhesins of *E. coli* (Klemm, 1994). Therefore, the presence of naturally occurring variability in the FimH adhesin raises important questions for the study of bacterial adhesins and lectins in general. It is important to understand whether it is physiologically critical for the naturally occurring 1M/3M-specific FimH variants of *E. coli* to maintain an intact 3M-binding capability or whether the acquisition of a strong monovalent 1M-binding property by FimH makes the multivalent 3M-specific binding a functionally redundant

property. Indeed, because any 3M receptor structure contains terminally exposed 1M residues, bacteria might be able to bind such receptors primarily or uniquely via a 1M-binding mode, i.e. without the need for a multivalent interaction.

In this report, we characterize novel functional variants of the FimH adhesin that are capable of strong 1M-specific binding, but lack the ability to mediate bacterial adhesion via 3M-like receptors in a specific manner. Such monovalent 1M-only binding variants provide important insights for understanding the structural basis and functional significance of naturally occurring variation in FimH specificity and microbial lectins in general.

Results

Binding of FimH–FocH hybrids to oligo- and monomannose receptor substrates

The architecture of the *E. coli* F1C fimbriae closely resembles that of the type 1 fimbriae (Riegman *et al.*, 1990). The equivalent of FimH in F1C fimbriae is FocH, which does not recognize mannoses but appears to mediate adhesion to vascular endothelial cells of the kidney and bladder via a glucosylceramide receptor (Khan *et al.*, 2000). FocH and FimH are similarly sized proteins with 36% sequence identity that can be readily exchanged between the two organelle systems, resulting in hybrid fimbrial organelles with receptor specificity defined by the adhesin (Klemm *et al.*, 1994; 1995). A set of chimeric FimH–FocH proteins was constructed recently with N-terminal segments of FimH from *E. coli* K-12 strain, ranging in size between 125 and 217 amino acids, and fused to sequential C-terminal segments of FocH (Fig. 1) (Knudsen and Klemm, 1998). *In trans* complementation of a plasmid-encoded *fimH*-null *fim* gene cluster with chimeric *fimH*–*focH* genes results in the expression of fimbrial rods that are structurally indistinguishable from fimbriae incorporating wild-type FimH–K-12 (Knudsen and Klemm, 1998).

Type 1-fimbriated *E. coli* strains expressing these FimH–FocH hybrid adhesins were tested for their ability to bind to a model 3M-like receptor substrate, BRB, and to a model 1M-like substrate, 1M-BSA. Three isogenic strains described previously (Sokurenko *et al.*, 1995; 1997) were used as controls. These strains express the following FimH variants of natural origin: (i) FimH from *E. coli* K-12 (FimH–K-12), which exhibits a strong 3M-specific and moderate 1M-specific binding phenotype (1M/3M binding ratio being within 0.25–0.35); (ii) FimH from the fecal *E. coli* isolate F-18 (FimH–F18), which exhibits a typical 3M-only binding specificity (1M/3M ratio \approx 0.05–0.15); and (iii) FimH from a uropathogenic *E. coli* isolate

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FimH 1  FACKTANGTAIPIGGGSANVYNLAPVNVVGQNL-VVDLSTQIFCHN-DY 48
FocH 1  LLCR--NNQTGGTQFGGDSRFNITLSPTVQYDKAITVLDLNLQVLVCQNEDA 49
      . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :
      : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :

FimH 49  PETITDYVTLQRGSAYGCVLSNFSG-----TVKYSGSSYPFFTSETP-- 91
FocH 50  SGQNYGYLVRVQGTGFSPLDAKTYGRLDFTNRLSGYSQTLPLQQDTKPT 99
      . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :
      : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :

FimH 92  RVVYNSRTDKPWPVALYLTVPSSAGGVAIKAGSLIAVLILRQTNVNSDD 141
FocH 100 EAYWQYGVWKPFPKMYLYPEPGVFGKLIHAGELVATVYVNVKFSMTMGQEA 149
      . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :
      : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :

FimH 142 --FQFVWNIYANNDDVVVPTGGCDVSARDVTVTLTPDYRG-SVPIPLIVYCA 188
FocH 150 GERNETWRFFYATNDVYIQGTGCRVSSNNVKVDLPSYPGGFVTVPLTVRCD 199
      . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :
      : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :

FimH 189 KSQNLGYLLSGTHADAGNSIFTNTIASFSAPGGVGVQLTRNGTTIPANNTV 238
FocH 200 QTQSVSYTLSSGSVTGGSNIVFANTAT-SGAGGVGVQLSDNAGLVPAQGPR 248
      . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :
      : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :

FimH 239 SLGAVGTSAVSLGLTANYARTG-GQVTAGNVQSIIGVTFVYQ 279
FocH 249 SLGGVQSSPVSLGLKASYALTGQASPTPGAVQSVINVTFSYN 290
      . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :
      : . . . . . : . . . . . : . . . . . : . . . . . : . . . . . :

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Fig. 1. Aligned amino acid sequences of the mature FimH-K-12 and FocH proteins. Gaps are included to obtain maximum fit. Identical residues are indicated by colons and functionally similar residues with periods. The residues 157–159 amino acids separating the N-terminal lectin domain from the C-terminal pilin domain of FimH are underlined (Choudhury *et al.*, 1999). Arrows indicate the C-terminal ends of FimH segments incorporated into the corresponding FimH–FocH hybrids (Knudsen and Klemm, 1998).

MJ2-2 (FimH–MJ2), which exhibits a distinct 1M/3M-specific binding phenotype (1M/3M ratio ≈ 0.7 –0.8).

Strains expressing FimH¹⁻²¹⁷-Foch²²⁸⁻²⁹⁰ and FimH¹⁻²⁰¹-Foch²¹³⁻²⁹⁰ hybrids bound to BRB and 1M-BSA at a level similar to the wild-type FimH-K-12 (Fig. 2), whereas strains expressing FimH¹⁻¹²⁵-Foch¹³⁴⁻²⁹⁰ and FimH¹⁻¹³⁹-Foch¹⁵¹⁻²⁹⁰ chimeras were unable to bind to either BRB or 1M-BSA. Strains expressing FimH-Foch chimeras that incorporate the 1-184, 1-170 and 1-158 regions of FimH mediated a strong, wild-type-like level of bacterial binding to the 3M-like substrate, BRB. However, unlike the FimH-K-12 parent, these hybrids acquired a very high affinity for the 1M-BSA substrate, with 1M/3M binding ratios within the range 0.9-1.0 (Fig. 2). These

hybrids became the focus of more detailed binding studies.

Sensitivity of the BRB and 1M-BSA binding to inhibition by methyl- α -D-mannopyranoside

The FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ hybrid was tested for its ability to recognize 3M-like and 1M-like substrates via, respectively, the multivalent 3M-specific and the monovalent 1M-specific mechanisms as described previously for naturally occurring FimH variants (Sokurenko *et al.*, 1997). 1M-specific binding differs from 3M-specific binding in being significantly more sensitive to the presence of soluble 1M, D-mannose and derivatives. Furthermore, the

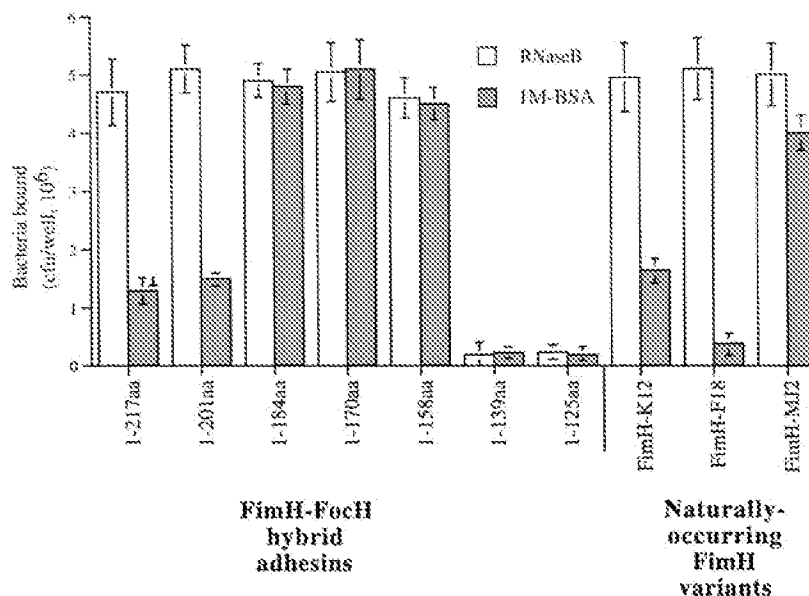


Fig. 2. BRB and 1M-BSA binding of isogenic *E. coli* strains expressing FimH–FocH hybrids and the naturally occurring variants -- wild-type FimH–K12, FimH–F18 (3M-only binding) and FimH–MJ2 (1M/3M binding). All binding is > 90% mannose inhibitable.

D-mannose inhibition profile of FimH variants binding to 1M-like substrates is a reflection of their relative ability to bind 1M-specific receptors.

FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰-mediated binding to 1M-BSA was highly sensitive to inhibition by methyl- α -D-mannopyranoside

(α mM). The α mM concentration causing 50% inhibition (IC_{50}) was 200- and 50-fold lower than that determined for the 1M-BSA binding mediated by FimH-K12 and FimH-MJ2 respectively (Fig. 3A). It should be noted that α mM inhibition of 1M-BSA binding of the FimH-F18 variant was

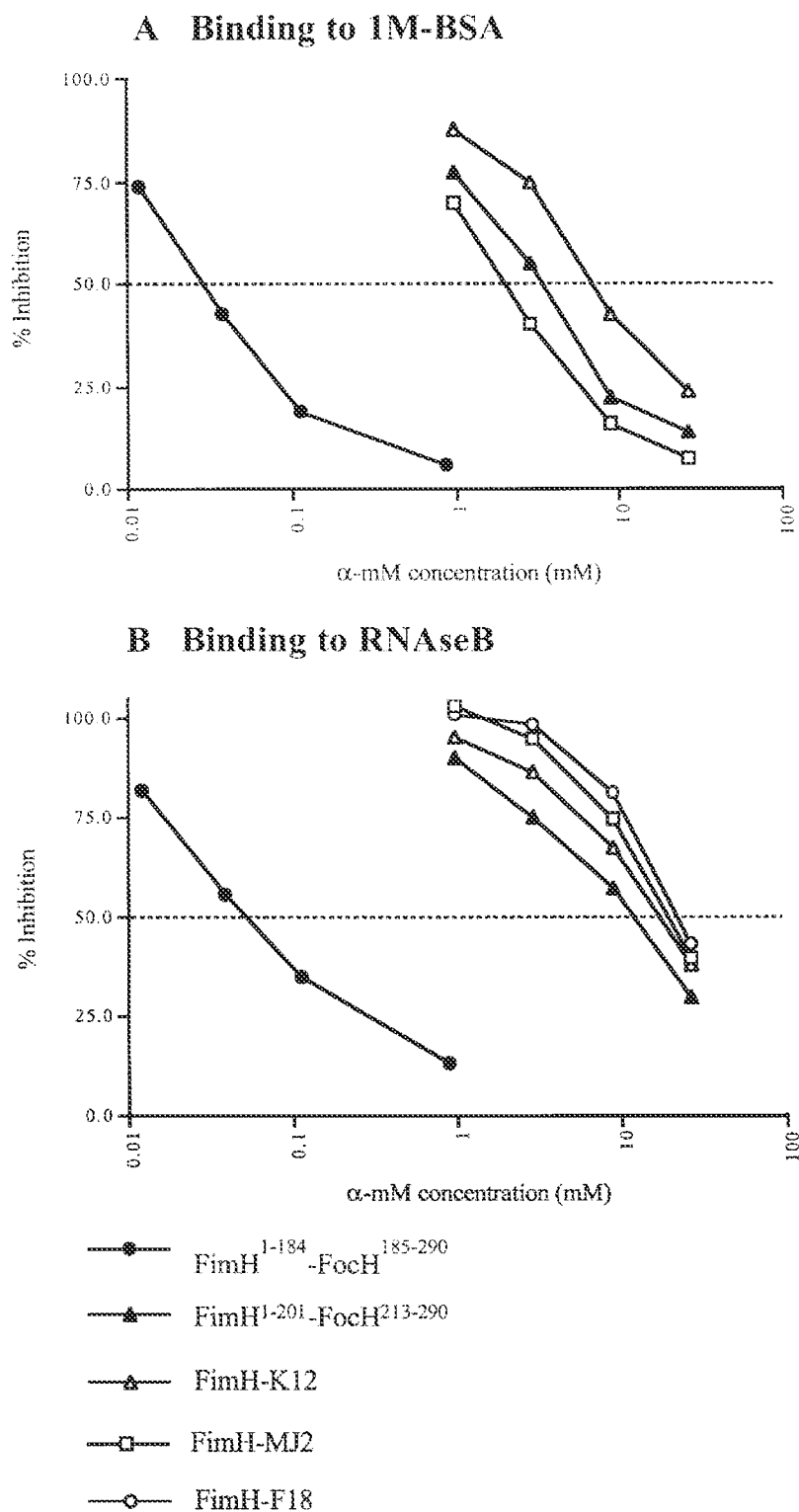


Fig. 3. Inhibition of the binding of isogenic *E. coli* expressing the FimH-FocH hybrids and naturally occurring FimH variants to BRB and 1M-BSA by α mM.

Table 1. Comparison of the eukaryotic cell-binding pattern of the *E. coli* strains expressing FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ or FimH¹⁻²⁰¹-FocH²¹³⁻²⁹⁰ hybrids and the naturally occurring FimH variants (all binding is > 90% mannose inhibitable).

Type 1 fimbrial adhesin variant	Monomannose-specific assays		Oligomannose-specific assays	
	J82 bladder cell binding (bacteria per cell)	RBC binding (titre)	Buccal cells binding (cells bound per well)	Yeast cell agglutination (titre)
FimH ¹⁻¹⁸⁴ -FocH ¹⁹⁶⁻²⁹⁰	65 ± 12	1:16	12 ± 7	1:1
FimH ¹⁻²⁰¹ -FocH ²¹³⁻²⁹⁰	19 ± 4	1:4	174 ± 14	1:16
FimH-K-12	15 ± 3	1:4	195 ± 12	1:16
FimH-F18	4 ± 2	1:2	180 ± 11	1:24
FimH-MJ2	52 ± 7	1:16	205 ± 24	1:16
FimH-MS260	53 ± 14	1:12	47 ± 13	1:2

not able to be measured reliably because of the very low 1M-BSA affinity of this variant. These results indicate that the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ hybrid exhibits a very distinct 1M-specific binding affinity. In parallel, we examined the α mM inhibition of the FimH-mediated binding to the 3M-like substrate, BRB (Fig. 3B). BRB binding of the strains expressing naturally occurring FimH variants was relatively insensitive to inhibition by α mM, which corresponded to the capability of these FimH variants to recognize 3M-like receptors via a multivalent 3M-specific mechanism. Surprisingly, the BRB binding mediated by the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ hybrid was almost as sensitive to the inhibition as the hybrid's binding to 1M-BSA, with the IC₅₀ being 250- to 350-fold lower than that determined for the strains expressing FimH-K-12, -F18 and -MJ2 variants. Therefore, in contrast to the naturally occurring FimH adhesins, the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin appears to be incapable of interacting with the 3M-like receptor via a multivalent 3M-specific mechanism. It is possible instead that the strong binding of this variant to the BRB substrate occurs primarily via monovalent 1M-specific interaction with individual mannosyl residues that are part of the 3M or other oligomannose structures of the BRB oligosaccharide moieties.

Strains expressing the FimH¹⁻¹⁵⁸-FocH¹⁶⁹⁻²⁹⁰ and FimH¹⁻¹⁷⁰-FocH¹⁸¹⁻²⁹⁰ hybrids had inhibition patterns very similar to that of the 1-184 amino acid hybrid (data not shown), whereas the FimH¹⁻²⁰¹-FocH²¹³⁻²⁹⁰ hybrid exhibited the wild-type, FimH-K-12 pattern (Fig. 3).

Eukaryotic cell-binding properties of the FimH-FocH hybrid adhesins

To define further the specificity of the chimeric FimH-FocH variants, we tested their ability to bind eukaryotic cells in a set of four assays that have previously been shown to define FimH receptor specificity (Sokurenko *et al.*, 1997; 1998). Yeast aggregation and buccal cell binding are both mediated by strains expressing either 3M only or 1M/3M-specific FimH variants. In contrast, rosette

formation of guinea pig red blood cells (RBCs) and adhesion to bladder epithelial tissue culture cells are specifically mediated only by strains expressing the 1M/3M-binding FimH variants.

Bacteria displaying the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin were capable of strong binding in the 1M-specific cell binding assays (Table 1), which was in accordance with the pronounced 1M-specific binding ability of the hybrid adhesin. However, this strain gave only marginally positive results in the 3M-specific assays, indicating that the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin is lacking 3M-specific receptor-binding capability. Obviously, although the strong monovalent-binding property of the hybrid adhesin provides bacterial binding to the purified 3M-like receptor, BRB, it cannot use 3M-specific receptors on the surface of yeast or buccal cells sufficiently to accomplish cell aggregation or binding respectively.

Again, strains expressing the FimH¹⁻¹⁵⁸-FocH¹⁶⁹⁻²⁹⁰ and FimH¹⁻¹⁷⁰-FocH¹⁸¹⁻²⁹⁰ hybrids had the same cell-binding pattern as the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ hybrid (not shown), whereas the FimH¹⁻²¹⁷-FocH²²⁸⁻²⁹⁰ hybrid had the wild-type-like FimH-K-12 pattern (Table 1).

Taken together, the adhesion patterns demonstrated by the 1-158, -170 and -184 amino acid FimH-FocH hybrids in the substrate-binding, mannose inhibition and cell-binding assays indicate strongly that this group of chimeric adhesins possesses a distinct ability to mediate bacterial binding via the monovalent 1M-specific mechanism, but lack the multivalent 3M-specific binding property. Such adhesins with a 1M-only binding phenotype represent a completely novel adhesin class that differs from the naturally occurring 3M-only and 1M/3M-specific FimH variants of commensal and uropathogenic *E. coli* respectively.

Probing the configuration of the receptor-combining site of a 1M-only specific adhesin

To compare the configuration of the receptor-binding pocket in the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin with that of

the wild-type FimH–K-12, we determined the relative inhibitory potencies of α mM and different complex mannose derivatives: (i) mannotriose (Man1–6Man1–3Man, representing the terminally exposed 3M structure of high-mannose type N-linked oligosaccharides); (ii) mannopentaose (Man1–3Man1–6[Man1–6Man1–3Man], representing an extended oligomannosyl component of certain high-mannose type N-linked oligosaccharides); and (iii) BRB, a soluble form of the glycoprotein used in our studies as the model 3M-like substrate. BRB primarily contains Man₅ and Man₆ high-mannose type oligosaccharides (Fu *et al.*, 1994). The inhibitory compounds were tested for their ability to inhibit binding of the FimH^{1–184}–FocH^{196–290} and the FimH–K-12-expressing bacteria to BRB, to which both FimH variants mediate strong binding, albeit via apparently different mechanisms.

Although the BRB binding mediated by the FimH^{1–184}–FocH^{196–290} hybrid adhesin was significantly more sensitive to inhibition by all three complex mannose derivatives than the wild-type FimH–K-12 (Table 2), for each adhesin, the relative inhibitory potency of the receptor analogues in comparison with α mM and each other was very similar and increased in the order: α mM < mannotriose < mannopentaose < BRB. These results suggest that, in both 1–184 amino acid FimH–FocH hybrid and the wild-type FimH–K-12, adhesin receptor binding is provided by an extended combining site with a similar conformation of receptor-interacting residues (see *Discussion*).

Invoking a 1M-binding FimH phenotype by a point mutation

The definition of a novel 1M-only FimH-binding subclass through the analysis of our FimH–FocH chimeras prompted us to screen for the presence of similar functional variants within the spectrum of FimH molecules altered by point mutations. For this purpose, we used a random mutant library of the FimH–K-12 adhesin created in a previous study (Schembri *et al.*, 2000).

Approximately 100 clones from the mutant library were

picked randomly and tested in the substrate and cell-binding assays described above. One clone, MS260, demonstrated a functional phenotype that was very similar to the 1M-only binding phenotype exhibited by the hybrid adhesins (Table 3). The MS260 strain demonstrated a very strong binding to 1M-BSA relative to BRB, with the binding to both substrates being highly sensitive to inhibition by α mM. Also, compared with the wild-type FimH–K-12 strain, MS260 was highly active in the 1M-specific cell-binding assays (i.e. binding to the bladder epithelial cells and rosette formation with guinea pig RBCs). At the same time, the MS260 bacteria demonstrated only weak activity in the 3M-specific cell-binding assays (i.e. yeast cell aggregation and buccal cell binding).

We determined the nucleotide sequence of the *fimH* gene of the MS260 clone. The FimH–MS260 adhesin sequence differed from the wild-type FimH–K-12 in a single amino acid position: glutamic acid in position 89 was replaced by lysine. Therefore, the E89K point mutation in the N-terminal part of the FimH molecule has the same functional impact on the binding profile as the replacement of the 158- to 185–279 amino acid C-terminal portion of FimH with a corresponding part of the FocH adhesin. Both types of structural alterations result in a 1M-only binding phenotype of the type 1 fimbrial adhesin.

Examination of the type 1 fimbrial morphology and FimH expression of the MS260 mutant clone

The morphology of the fimbriae expressed by the MS260 clone was examined by electron microscopy. Fimbrial length, shape and number per cell were similar between the MS260 and the wild-type FimH–K-12 clones (Fig. 4). We also compared the level of FimH expression on the bacterial surface with an anti-FimH antiserum (Fig. 5). There was essentially no difference between the anti-FimH reactivity of the bacterial cells expressing either the FimH–MS260 mutant variant or the wild-type FimH–K-12 adhesin. Therefore, the E89K substitution that invokes the

Table 2. Inhibitory potency of the different mannose compounds against BRB binding of the *E. coli* strains expressing the wild-type FimH–K-12 and FimH^{1–184}–FocH^{196–290} hybrid.

Inhibitor compound: Name; mol. weight (in Da) and structure	IC ₅₀ mM (potency versus α mM)	
	FimH–K-12	FimH ^{1–184} –FocH ^{196–290}
α mM; 194	12.0 \pm 2.0 (1)	0.045 \pm 0.005 (1)
Mannotriose; 504	4.1 \pm 0.6 (2.9)	0.023 \pm 0.004 (2.0)
Mannopentaose; 829	2.0 \pm 0.3 (5.9)	0.009 \pm 0.002 (5.1)
Bovine BRB; 15 500 (major oligosaccharide component)	1.0 \pm 0.2 (12.0)	0.0055 \pm 0.001 (8.2)

1M-only binding phenotype of the FimH adhesin does not lead to an aberrant fimbrial morphology or FimH expression on the surface of bacteria.

Discussion

The type 1 fimbrial adhesin of *E. coli*, FimH protein, is folded into a 1–156 amino acid N-terminal lectin domain containing a receptor binding site and a 160–290 amino acid C-terminal pilin domain that interacts with the molecular chaperone, FimC, and provides for incorporation of the adhesin into the fimbrial shaft (Choudhury *et al.*, 1999). As with most of the lectins, the FimH adhesin exhibits a multivalent type of binding and is capable of recognizing oligomannose receptors containing unsubstituted 3M structures in a specific manner (Sokurenko *et al.*, 1997). However, FimH variants expressed by most of the uropathogenic isolates, but not by commensal strains, are also capable of a relatively strong monovalent 1M-specific interaction with mannosyl residues (Sokurenko *et al.*, 1995; 1997). Such dual 1M/3M specificity provides the bacteria with a selective edge in the colonization of the urinary bladder (Sokurenko *et al.*, 1998). In this study, we show that it is possible to separate the monovalent 1M-binding property of the FimH adhesin from the multivalent 3M-binding capability. We demonstrate that (i) the *E. coli* FimH adhesin can be converted into a 1M-only binding lectin by a structural alteration of either the shaft-associated pilin domain or the receptor-binding lectin domain of the adhesin; (ii) both the 1M- and 3M-specific receptor interaction of the FimH adhesin is provided by a single binding pocket of an extended structure; and (iii) the 3M-binding specificity of the FimH adhesin is a physiologically essential property that cannot be functionally compensated by a strong 1M-binding specificity.

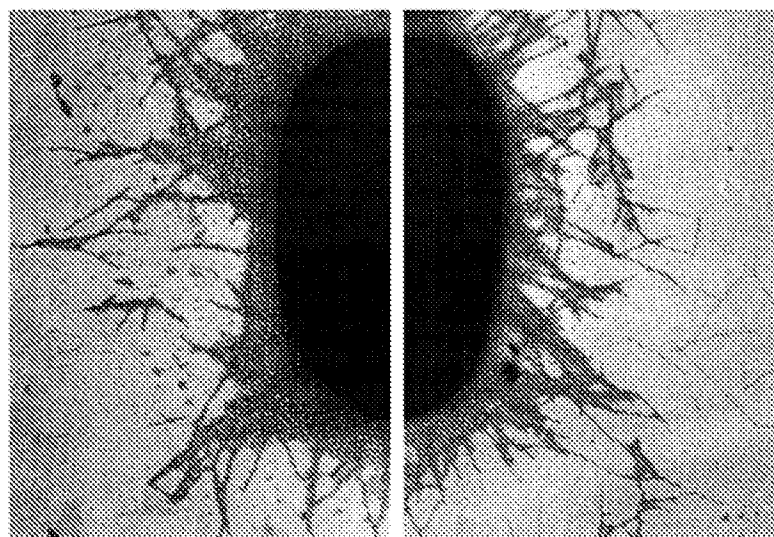
Studies reported here were primarily performed using chimeric FimH–FocH hybrid adhesins that were partially characterized previously (Knudsen and Klemm, 1998). FocH is the adhesive subunit of the glucosylceramide-specific type 1C (F1C) fimbriae of *E. coli* (Khan *et al.*, 2000). The active form of FocH or the FimH–FocH hybrids

can be incorporated into type 1 fimbriae in place of the FimH subunit without affecting fimbrial morphology (Klemm *et al.*, 1994; Knudsen and Klemm, 1998). It has been shown that bacteria expressing FimH–FocH hybrids with a 1–201 amino acid or larger portion of the FimH adhesin have preserved the capability of agglutinating yeast cells in a mannose-sensitive manner. Interestingly, it was shown that, although bacteria expressing FimH–FocH hybrids incorporating 1–158 amino acid to 1–184 amino acid portions of FimH were unable to mediate yeast cell agglutination, they could bind to D-mannose-coated beads.

The novel observations in this study were obtained by characterizing the multivalent 3M- and monovalent 1M-binding properties of the FimH–FocH hybrid adhesins. The hybrid adhesins incorporating residues 1–201 and 1–217 of FimH exhibited a distinct 3M specificity and moderate 1M specificity similar to the wild-type FimH variant of *E. coli* strain K-12. This was not surprising as the fused C-terminal portion of FocH is highly homologous to the corresponding FimH region (see Fig. 1), which is primarily involved in interaction with the molecular chaperone FimC (Choudhury *et al.*, 1999). In contrast, FimH–FocH hybrids incorporating 1–139 amino acids (or less) of FimH were completely non-functional in any of the tests. It was demonstrated previously that structural integrity of the 133–141 amino acid region of the FimH lectin domain (which shows very low homology with the corresponding FocH region) is critically important for FimH function and constitutes part of the receptor-binding pocket (Schembri *et al.*, 1996; Choudhury *et al.*, 1999). The most important results of this study were obtained using the 1–158 amino acid, 1–170 amino acid and 1–184 amino acid FimH–FocH hybrid adhesins. Compared with the wild-type FimH–K-12, these hybrids demonstrate a significantly improved ability to bind immobilized 1M-BSA substrate and mediate bacterial adhesion in the 1M-specific cell-binding assays (binding to RBCs and adhesion to uroepithelial tissue culture cells). At the same time, binding of these hybrids to the 3M-containing substrate BRB was 200-fold more sensitive to inhibition by a 1M derivative, α mM, than was the multivalent

Table 3. Comparison of the eukaryotic cell-binding pattern of the *E. coli* strains expressing FimH–MS260 and FimH–K-12 (All binding is > 90% mannose inhibitable).

FimH variant	1M-BSA/BRB binding ratio	Inhibitory activity of α mM (IC ₅₀ , mM)		1M-specific assays		3M-specific assays	
		1M-BSA binding	BRB binding	J82 cell binding (bacteria per cell)	RBC binding (titre)	Buccal cell binding (cells per well)	Yeast cell aggregation (titre)
FimH-MS260	0.94	0.025	0.04	53 ± 14	1:12	47 ± 13	1:2
FimH-K-12	0.35	8.0	20.0	12 ± 2	1:4	185 ± 15	1:16



FimH-MS260

FimH-K12

Fig. 4. Electron microscope photographs of type 1-fimbriated cells of the *E. coli* strains expressing the FimH-MS260 mutant and the wild-type FimH-K-12.

3M-specific binding mediated by the FimH-K-12 and other naturally occurring FimH variants. Furthermore, bacteria expressing these hybrid adhesins were unable to mediate adhesion in the cell-binding assays that are readily mediated by 3M-only or 3M/1M-specific FimH variants (yeast aggregation and human buccal cell binding). Therefore, we propose that the 1–158 amino acid, 1–170 amino acid and 1–184 amino acid FimH–FocH hybrids exhibit a novel, 1M-only specific binding phenotype and have essentially lost the capability for the multivalent 3M-specific FimH–receptor interaction. Interestingly, the 1–156 amino acid lectin domain of FimH is fully incorporated into the hybrid structures and, therefore, structural alteration of the pilin domain is responsible for the functional modification. The pilin domain is thought to provide incorporation of the FimH subunit into the fimbrial shaft (Choudhury *et al.*, 1999). However, fimbrial shaft-induced alterations in FimH specificity have been reported in previous studies, in which expression of the *E. coli* FimH adhesin within type 1 fimbriae of heterologous species resulted in the species-specific functional changes in the adhesin (Madison *et al.*, 1994; Thankavel *et al.*, 1999). Therefore, the 1M-only binding phenotype of the corresponding FimH hybrids could be induced by an altered conformation of the FimH–FocH hybrid subunits within the fimbrial shaft, i.e. as a result of certain distal effects on the binding site of FimH, located within the lectin domain. One can speculate, for example, that the functional properties of the FimH lectin domain could be affected by the mode of its interaction with the FimH pilin domain or other fimbrial subunits, i.e. by the quaternary structure of FimH. Alternatively, the acquisition of a strong monovalent binding capability combined with the loss of a multivalent

binding property could result from direct alteration of an additional, structurally separate binding pocket of unknown nature located within the pilin domain.

A common way to compare the configuration of receptor-binding pockets of lectins is to determine relative inhibitory capabilities of different receptor analogues with

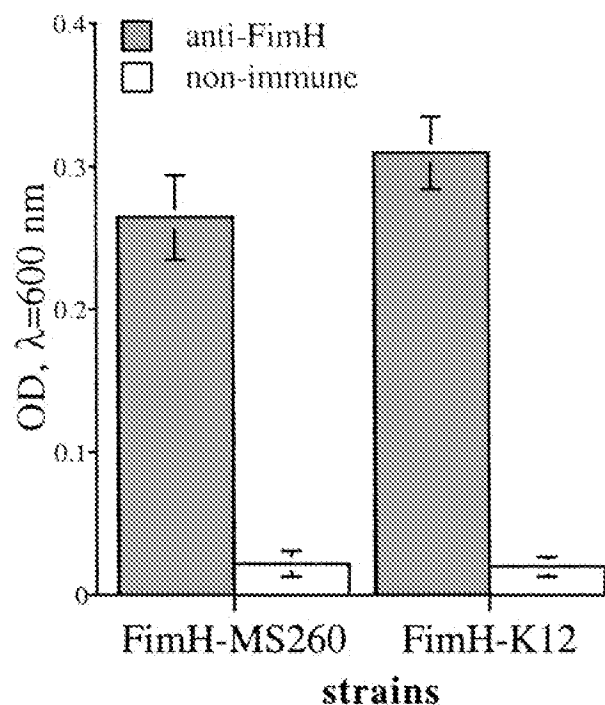


Fig. 5. Reactivity of the anti-FimH and non-immune serum with a plastic-immobilized bacteria of the *E. coli* strains expressing the E89K FimH mutant and the wild-type FimH-K-12.

defined structures (Firon *et al.*, 1984; Gupta *et al.*, 1996). To compare the configuration of the 1M-only binding site within the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin and of the 3M binding site within the wild-type FimH-K-12 variant, we used as inhibitors four mannose derivatives of increasingly complex structure – α mM, 3M, mannopentaose and a receptor substrate itself, BRB, containing high-mannose type oligosaccharides. As was expected, the 3M-specific binding to BRB mediated by the FimH-K-12 adhesin is provided by a combining site of a multivalent structure (i.e. containing multiple subsites), because inhibitors with a more complex structure demonstrate a higher affinity of interaction with the adhesin. In fact, the 3M-specific combining site of FimH appears to have a better fit for the mannopentaose and high-mannose oligosaccharides than for the less complex 3M-like receptor structure. This finding is in agreement with previous studies, which have shown that compounds such as methyl-mannotriose and certain high-mannose type oligosaccharides are significantly more potent than D-mannose or α mM in blocking yeast or RBC agglutination by the type 1-fimbriated *E. coli* (Firon *et al.*, 1984; Lindhorst *et al.*, 1998). Inhibition analysis of the 1M-only specific binding to BRB mediated by the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ hybrid indicates that the configuration of its binding site resembles the multivalent configuration of the 3M binding site within FimH-K-12, because the relative inhibitory activities of the mannose derivatives are similar for both types of adhesin. As it is rather unlikely that there are two separate binding sites within the FimH molecule that have such similar extended structures, the multivalent 3M-specific binding and the monovalent 1M-only specific binding are obviously provided by a single site, or structurally overlapping binding pockets, within FimH. It is possible that, within the combining site of the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ hybrid adhesin, conformation of only one or few subsite(s) is affected, which resulted in a dramatically increased affinity of interaction with individual mannose residues. The presence of a structurally distinct, monomannose binding subsite within a multivalent combining site is known from detailed crystallography studies of such mannose-specific lectins as concanavalin A and a lectin from *Dioclea grandiflora* (Rozwarski *et al.*, 1998). However, the exact structure of the combining site within FimH is unknown, because the binding pocket identified by X-ray study of the FimH-FimC complex was configured using a relatively small compound, cyclohexylbutanol-*N*-hydroxyethyl-D-glucamide (C-HEGA), which rather distantly resembles the D-mannose structure (Choudhury *et al.*, 1999).

The multivalent configuration of the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin binding site is somewhat surprising, as this adhesin appears to be incapable of multivalent binding to surface-immobilized oligomannose receptors. Obviously, although the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin

interacts with the soluble form of oligomannose compounds in a multivalent manner, the strong 1M binding subsite might interfere functionally with the multivalent interaction between the adhesin and immobilized mannosylated receptors. Another possibility is that interaction between the bacterial surface adhesins and surface-immobilized receptors involves certain conformational changes in either adhesin or receptor molecules that are absent when the receptor compounds are in soluble form.

Because FimH¹⁻²⁰¹-FocH²¹³⁻²⁹⁰ hybrid adhesins exhibit the wild-type-like FimH phenotype, the 1M-only binding phenotype of the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ adhesin obviously resulted from the replacement of the 185–201 amino acid region of the FimH pilin domain by the corresponding FocH region. However, when we screened a random mutant library of FimH for clones with a 1M-only binding phenotype, we identified a mutant containing a single amino acid replacement, E89K, in the FimH lectin domain. The location of the E89K mutation within the lectin domain of FimH indicates that the 1M-only specific phenotype can also be induced without any alteration in the pilin domain. This provides additional evidence that the loss of the 3M-binding specificity is unlikely to result from the impairment (and therefore the existence) of a receptor binding site within the pilin domain of FimH. Because the combined evidence implies that the 1M-only specific phenotype of the FimH¹⁻¹⁸⁴-FocH¹⁹⁶⁻²⁹⁰ and corresponding hybrids results from a distal conformational effect on the single binding site within FimH, the E89K mutation might invoke the 1M-only specific phenotype in an indirect manner as well. The hypothesis of a distal effect of the E89K mutation on binding site conformation is also supported by the fact that this mutation maps to the 'bottom' of the β -barrel-shaped lectin domain of FimH, i.e. to the region opposite the location of the binding site identified by X-ray crystallography (Choudhury *et al.*, 1999) (see Fig. 6). Because the 'bottom' region of the lectin domain seems to be accessible for possible interdomain or intersubunit interactions, the location of the E89K mutation is also in line with the notion expressed above that the structural replacements or mutation could affect the FimH quaternary structure, which might, in turn, be important for the integrity of FimH function. Interestingly, naturally occurring mutations that confer the dual 1M/3M-specific phenotype map into the same 'bottom' region of the FimH lectin domain (Schembri *et al.*, 2000). Also, similar to the naturally occurring mutations, expression of the E89K mutant does not lead to an aberrant morphology of fimbriae or to a significant change in the amount of surface-expressed FimH (Sokurenko *et al.*, 1994; 1997). Therefore, it is possible that the uropathogenicity-adaptive 1M/3M-binding phenotype of FimH and the 1M-only specific phenotype are induced by conformational changes of a similar nature, i.e. by distally affecting the

properties of a single binding site of FimH. It is also possible, however, that the point mutations alter an alternative binding site(s), of as yet unknown nature, located in the 'bottom' region of the FimH lectin domain.

Although the monovalent 1M-only specific phenotype can be invoked by a point mutation in FimH, no such variants have been identified so far among the naturally occurring *fimH* alleles. The 1M-only specific FimH variants were unable to mediate buccal cell binding, which seems to depend on an intact multivalent 3M-specific binding property of FimH. Therefore, this type of adhesin, if expressed naturally, might be incapable of providing the transient oropharyngeal colonization important in *E. coli* interhost transmission (Bloch *et al.*, 1992). Consequently, the 1M-only binding phenotype is likely to be inferior to the 3M-only or dual 1M/3M-specific phenotypes and would be strongly selected against in nature. We therefore speculate that, although an increased 1M-binding capability of the FimH lectin is highly adaptive for the uropathogenic *E. coli*, its acquisition does not outweigh the physiological importance of the 3M-specific binding property of type 1 fimbriae. This explains why the 3M-binding specificity is a conserved property among naturally occurring FimH alleles identified so far and, consequently, why the 1M-only binding variants are not found among wild-type *E. coli* isolates. The phenomenon of functional inferiority of the 1M-only specific FimH adhesin provides an important insight into possible physiological consequences that might result from the loss of a lectin's ability to recognize oligosaccharide ligands in a specific manner. Our study has demonstrated that even a strong

monovalent binding capability of a lectin might be insufficient for providing certain types of cell-cell interactions that are readily mediated by the multivalent oligosaccharide-specific binding. The addition of the 1M-only binding FimH mutants to the collection of previously defined 3M-only and 1M/3M-binding FimH would help us in understanding the structure-functional properties of type 1 fimbriae and of other types of bacterial adhesins and lectins in general.

Experimental procedures

Bacterial strains and plasmids

Construction of type 1-fimbriated *E. coli* strains displaying hybrid FimH-FocH adhesins has been described previously (Knudsen and Klemm, 1998). Briefly, using the *fimH* allele of *E. coli* K-12 as wild type, various sequential *fimH*-*focH* fusions were constructed and inserted into pBR322 (Ap^r) under the *tet* promoter, and the hybrid-expressing plasmids were transferred into *fimH*-null strain VL751(pPKL115, Cm^r) for phenotype expression. Isogenic strains expressing *fimH* alleles subcloned from a fecal *E. coli* isolate, F-18, or a uropathogenic isolate, *E. coli* MJ2-2, have been described previously (Sokurenko *et al.*, 1995; 1997). Briefly, the *fimH* alleles were subcloned into the pACYC184-based vector pGB2-22 (Cm^r) under the control of the *bla* promoter, and the *fimH*-expressing plasmids were transferred into *fimH*-null strain AAEC191A(pPKL114, Ap^r) for phenotype expression. A random mutant library of FimH-K-12 was constructed by polymerase chain reaction (PCR) mutagenesis as described previously (Schembri *et al.*, 2000). Briefly, a 656 bp *KpnI*-*HincII* region of the *fimH* gene was mutagenized by PCR amplification under suboptimal conditions; the mutant *fimH* genes were expressed in the pUC19-based vector pMAS1 (Ap^r), which was transferred into *fimH*-null strain S1918(pPKL115, Cm^r) for phenotype expression.

Reagents

Mannosylated BSA (1M-BSA) was obtained from EY Laboratories. Ribonuclease B from bovine pancreas (BRB) and all other reagents were purchased from Sigma Chemical. Anti-FimH antiserum was provided by Dr Scott Hultgren (Washington University, St Louis, MO, USA).

Receptor adhesion assays

1M-BSA is a model synthetic substrate for characterization of the 1M-specific binding of the FimH adhesin. BRB is a glycoprotein containing oligosaccharide moieties with high-mannose type structures and represents a model substrate for the 3M-specific binding of FimH. Assays of bacterial adhesion to immobilized 1M-BSA or BRB bound to 96-well plates or to epithelial cells in 8-well tissue culture chamber slides (Nunc) were carried out as described previously (Sokurenko *et al.*, 1997; 1998). Briefly, glycoproteins were dissolved at 20 µg ml⁻¹ in 0.02 M bicarbonate buffer, and 100 µl aliquots were incubated in microtitre wells for 1 h at

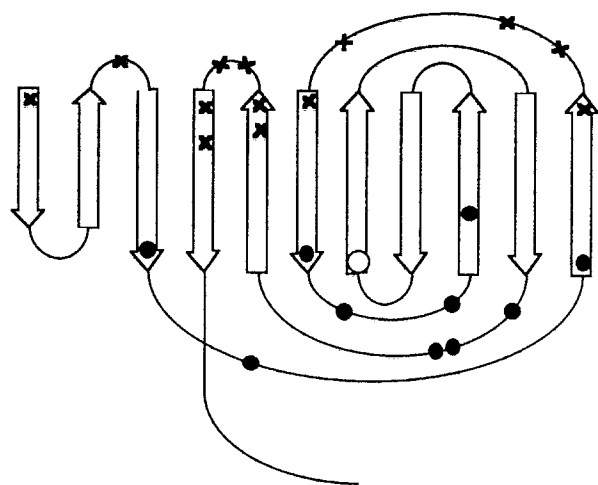


Fig. 6. β -Sheet topology diagrams of the lectin domain of FimH (Choudhury *et al.*, 1999). Although the lengths of the sheets and loops do not reflect the actual size, the relative position of the labelled residues is indicated accurately. The open circle indicates the position of the E89K mutation. The filled circles indicate the position of point mutations inducing the dual 1M/3M-binding phenotype (for details, see Schembri *et al.*, 2000). The crosses indicate residues interacting with the receptor analogue, C-HEGA.

37°C. The wells were then washed three times with 0.01 M phosphate-buffered saline (PBS, pH 7.2) and quenched with 0.1% BSA. ³H-thymidine-labelled bacteria (5×10^7 cfu) were added in 0.1% BSA in PBS, incubated for 40 min at 37°C, and the wells were then washed with PBS. The density of bacteria used provides an under-plateau dose of bacterial cells allowing quantitative comparison of the receptor-binding capabilities of different *E. coli* strains. Inhibitor titration of bacterial binding to immobilized 1M-BSA and BRB with methyl α -D-mannose and other mannosylated compounds was carried out essentially as described previously (Sokurenko *et al.*, 1995; 1997). The solution of BRB used for the inhibitory studies was dialysed extensively to remove possible low-molecular-weight components. All comparative studies were performed in parallel.

Eukaryotic cell-binding studies

Cell-binding assays were performed as described previously (Sokurenko *et al.*, 1997; 1998). Briefly, yeast aggregation assays were performed by mixing equal amounts of serially diluted bacterial suspensions (starting from OD₅₄₀ = 1.0) and a 1% suspension of baking yeast in U-bottomed microtitre plate wells under shaking conditions. Binding to guinea pig red blood cells (RBCs) was measured by the ability of the serially diluted bacteria to form rosettes of 1% suspension of RBCs under static conditions in the U-bottomed wells. Buccal epithelial cell binding and binding inhibition experiments were performed using an inverted adhesion assay described previously (Sokurenko and Hasty, 1995; Sokurenko *et al.*, 1998). Adhesion to the J82 urinary bladder epithelial cell line was determined by enumerating bound bacteria visualized by light microscopic examination of stained samples (Sokurenko *et al.*, 1995; 1997; 1998).

Electron microscopy

Bacteria were absorbed to coated grids and stained with 0.5% phosphotungstic acid as described previously (Sokurenko *et al.*, 1997; 1994).

Reactivity with anti-FimH antibodies

Anti-FimH or control non-immune rabbit serum was diluted 1:1000 in 0.1% BSA-PBS and incubated for 1 h with 2×10^6 bacterial cells immobilized on plastic in microtitre plate wells. The number of plastic well-immobilized cells was determined to be equal between different strains using ³H-thymidine-labelled bacteria. The reactivity of the bacterial cells with the anti-FimH and control antibodies was assessed using horseradish peroxidase-labelled anti-rabbit IgG antibodies.

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